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Antiherpesvirus Action of Atropine

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The antimuscarinic compound atropine shows an antiherpesvirus effect as measured by the protection of the cell monolayer and the reduction of the formation of new infectious virus. Atropine at a concentration of 200 μg/ml blocks the production of new infectious herpes simplex virus-type 1 virions. At that concentration, it has almost no effect on cellular or viral protein synthesis even when atropine is present from the beginning of the infection. The glycosylation of viral proteins is almost totally blocked when atropine is added. Although the viral proteins are underglycosylated, the formation of new herpes simplex virus type 1 virions takes place. The virions formed in the presence of atropine are noninfectious, and their protein composition, as assessed by labeling with [^{35}S]methionine, is similar to that of the control, except that they are not glycosylated.

The search for new antiherpesvirus agents is widening at present. A number of compounds have been found in the last few years that show a selective inhibitory effect against herpesvirus functions, with moderate toxicity to cells (1, 2). Most of those antiherpesvirus agents, such as acyclovir, bromovinyl deoxyuridine, and phosphonoformate (1, 2, 6), act at the level of nucleic acid synthesis. The first two compounds show a high antiviral index, but since they are nucleoside analogs, they should be used with caution because of their potential mutagenic and teratogenic effects. New antiherpesvirus compounds acting on other viral functions need to be found. These new inhibitors in turn would be very useful to study in more detail the different steps of virus replication.

Looking for new compounds with antiherpesvirus activity, we found several that blocked virus replication selectively, with a low toxicity against culture cells (B. Alarcón, J. C. Lacal, J. M. Fernández-Sousa, and L. Carrasco. Antiviral Res., in press). Among those compounds, atropine was active not only against herpes simplex virus type 1 but also against other enveloped viruses (Alarcón et al., in press). Yamazaki and Tagaya (11). also found a similar antiviral effect of atropine and caffeine. Therefore, we decided to investigate the mechanism of action of atropine against HSV-1. The results are presented in this paper.

MATERIALS AND METHODS

Cells and viruses. HeLa cells were grown in Dulbecco modified Eagle medium (GIBCO Laboratories) supplemented with 10% newborn calf serum (GIBCO). HSV-1 strain KOS, kindly given to us by E. de Clerq (Leuven), was grown and titrated in African green monkey kidney cells (Vero cells).

Estimation of the cytopathic effect. Monolayers of HeLa cells were infected with HSV-1 or HSV-2 at a multiplicity of 0.5 PFU per cell in the presence or absence of atropine. At 48 h after incubation, the cell monolayer was examined under a phase-contrast microscope. The level of translation was also determined by 1 h of incubation with 1 μCi of [^{35}S]methionine (1,100 Ci/mmol; Radiochemical Centre) per ml. The trichloroacetic acid-precipitable radioactivity was measured in an Intertechnique liquid scintillation counter.

Measurement of production of HSV-1 infectious units. Monolayers of HeLa cells were infected with HSV-1 at a multiplicity of 0.5 or 10 to analyze the reduction of infectious units in several cycles or in one cycle of replication. The infected cells were collected at 48 or 24 h postinfection, respectively. Atropine was added at the beginning of infection. Infected cells were disrupted by three freeze-thawing cycles. The infectious viruses produced were estimated by the standard plaque assay.

Analysis of proteins by polyacrylamide gel electrophoresis. After incubation of the cells in medium without methionine in the presence of 5 μCi of [^{35}S]methionine per ml for the period of time indicated in each experiment, the medium was removed, and the cell monolayer was washed with 1 ml of saline phosphate buffer; the cells were dissolved in 0.1 ml of sample buffer (62.5 mM Tris [pH 6.8], 2% sodium dodecyl sulfate, 0.1 M dithiothreitol, 17% glycerol). Each sample was sonicated to reduce viscosity and heated to 90°C for 5 min. Samples (7 μl each) were analyzed by polyacrylamide gel electrophoresis with 15% acrylamide gels. The gels were run overnight at 30 V, and fluorography was carried out with 2,5-diphenyloxazole-dimethylsulfoxide (20% [wt/wt]). The dried gels were exposed by using XS-5 X-ray films (Eastman Kodak Co.).

Analysis of glycoproteins. Cells were incubated for the period of time indicated in each experiment in medium without glucose supplemented with 10 mM sodium pyruvate in the presence of 10 μCi of D-[6-^{3}H]glucosamine (35 Ci/mmol; Radiochemical Centre), per ml and without serum. Labeling and sample preparation for electrophoresis were carried out as described above.

Electron microscope analyses. HeLa cells were infected with HSV-1 at a high multiplicity (10 PFU per cell) in the presence or absence of atropine. At 18 h postinfection, the cells were harvested, disrupted as described, and centrifuged at low speed. Supernatants were adsorbed to grids, and adhered particles were stained with 2% phosphotungstic acid at neutral pH. Microscopic examination was carried out in a JEOL electronic microscope.

Purification of radiolabeled HSV-1 particles. HeLa cells were infected with HSV-1 at 10 PFU/ml, and atropine was added. At 4 h postinfection [^{35}S]methionine (5 μCi/ml) or [^{3}H]glucosamine (20 μCi/ml) was added. At 16 h postinfection, the cells were harvested, disrupted as described, and then centrifuged at 15,000 × g for 10 min. Supernatants were added onto 1 ml of 10% sucrose in saline phosphate buffer and centrifuged at 40,000 rpm for 1 h in a Sorvall SW50 rotor. Pellets were dissolved in 50 μl of sample buffer, and electrophoresis was done as described above.

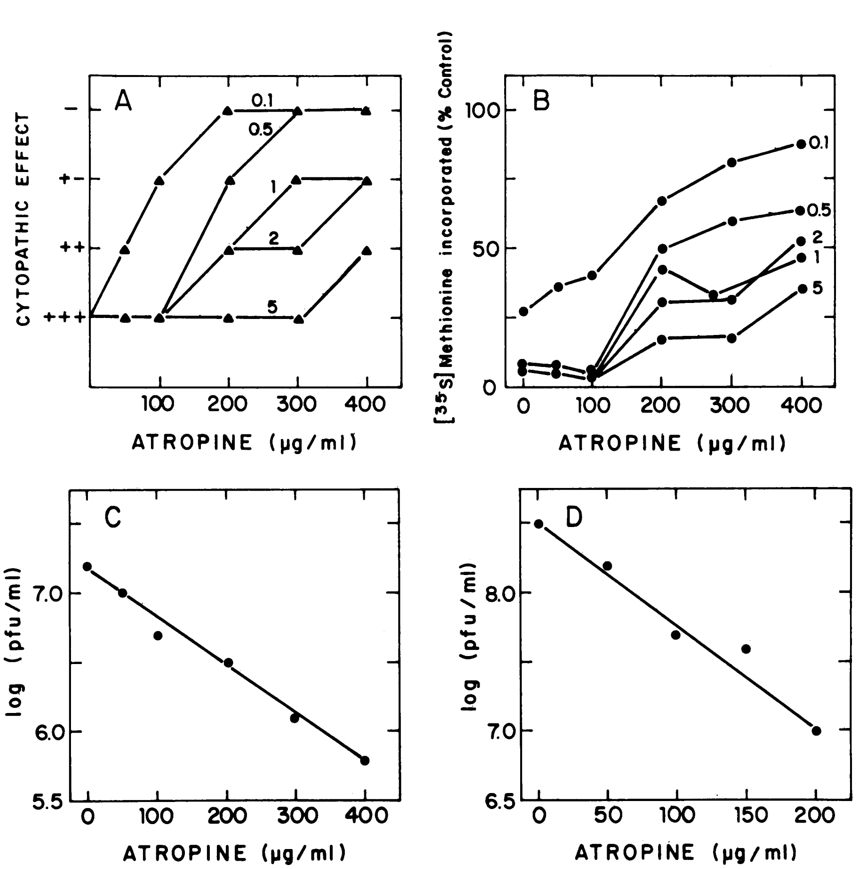

FIG. 1. Antiherpesvirus effect of atropine. (A) CPE in HSV-1-infected HeLa cells, 48 h postinfection. HeLa cells were grown and infected as described in the text. The symbols −, +−, ++, and +++ represent CPE on a progressive scale, varying from no CPE (−) to maximum CPE (+++). The numbers in the panel represent the multiplicities of infection (PFU per cell) used. (B) Protein synthesis in HSV-1-infected HeLa cells, 48 h postinfection. The numbers represent the multiplicities of infection used. (C) Production of infectious HSV-1 particles in a single-step growth cycle in the presence of several concentrations of atropine. The procedure used is described in the text. (D) Production of infectious HSV-1 particles in several growth cycles in the presence of different concentrations of atropine.

RESULTS

Antiherpesvirus action of atropine. First, we analyzed the atropine protection of the cytopathic effect of HeLa cells induced by HSV-1 under different multiplicities of infection (Fig. 1A). Atropine protected the cytopathic effect less efficiently when high multiplicities of infection of HSV-1 were used, but if the virus had to replicate several times to induce the destruction of the cell monolayer, then atropine was effective at concentrations of 50 to 100 µg/ml. These concentrations agree well with the results previously reported on the inhibition of HSV-1 and influenza plaque units by atropine (11). Figure 1B shows the level of protein synthesis in those cells incubated for 48 h in the presence of various concentrations of atropine. It is worth mentioning the relatively low toxicity of atropine in control cells, since not only did the cell monolayer remain intact, as observed under the microscope, (Fig. 1A), but also the cells synthesized proteins at control levels even in the presence of 400 µg of atropine per ml. Besides, the cells which were infected with HSV-1 at low multiplicity synthesized proteins to almost control levels in the presence of 400 µg of atropine per ml. In a similar experiment, this same effect was not observed with drugs like acyclovir or bromovinyl deoxyuridine, where the HSV-1-infected cells were morphologically protected, as observed under the microscope, but did not synthesize proteins after 48 h of treatment, even when the lowest multiplicity of infection of HSV-1 was used (Alarcón et al., in press). We have no obvious explanation for such behavior of the HSV-1-infected cells regarding the effects of acyclovir or bromovinyl deoxyuridine. One possibility is that since virus-infected cells are able to phosphorylate these compounds by means of the viral thymidine kinase, they might interphere with protein synthesis after long incubation periods. The inhibition of the formation of new infectious HSV-1 by atropine in a single-step or multiple-step growth cycle is shown in Fig. 1, panels C and D, respectively. Atropine (100 µg/ml) diminished by 1 logarithm the production of HSV-1 PFU and some effect was already observed at 50 µg/ml. On the other hand, at 200 µg/ml, which is approximately equivalent to 1 mM atropine, there was a significant reduction in the formation of PFU. These concentrations compare well with the antiviral potency of other antiherpesvirus compounds which are not nucleoside analogs, such as phosphonoformate and 2-deoxyglucose (Alarcón et al., in press).

Molecular mechanism of action. Once we determined the antiherpesvirus potency of atropine, the action of this compound on protein synthesis was investigated. We first examined the way different concentrations of atropine affected translation in HeLa cells that were either mock infected or infected with HSV-1 at a multiplicity of infection of 10. Figure 2 shows that concentrations of atropine up to 200 µg/ml did not inhibit either viral or cellular translation. Inhibition of some viral proteins was apparent in the 6- to 8-h

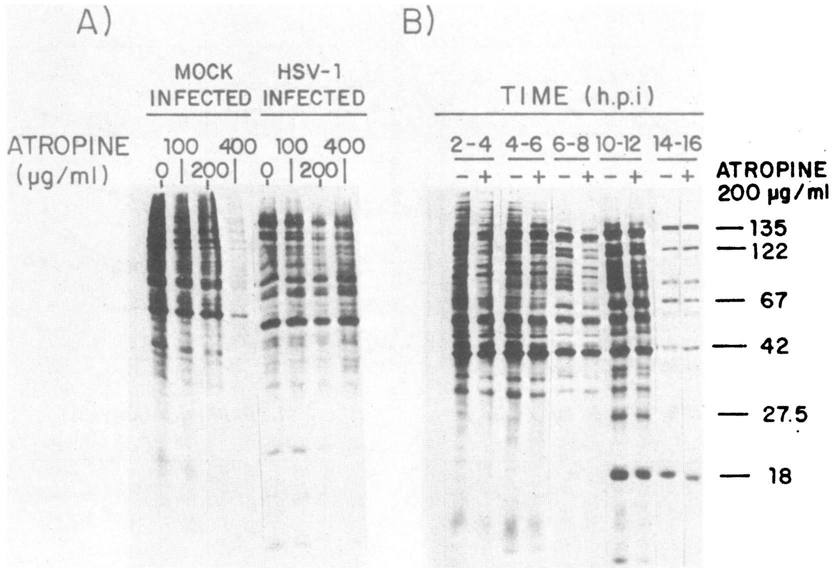

FIG. 2. Effect of atropine in protein synthesis. (A) Protein synthesis in mock-infected or HSV-1-infected cells treated with several concentrations of atropine. Atropine was added from the beginning of infection to 14 h postinfection. Afterwards, the cells were incubated with [^{35}S]methionine for 2 h as described in the text. (B) Time course of protein synthesis in HSV-1-infected cells in the presence or absence of 200 μg of atropine per ml. Proteins were labeled by two hourly pulses with [^{35}S]methionine as described in the text. Numbers on the right indicate the molecular weights (10^3) of some HSV-1-induced polypeptides.

pulse, but it was not apparent later. At 400 μg/ml, a slight inhibition in total protein synthesis was observed. However, as noted above, 200 μg of atropine per ml caused a 90% reduction in the production of new infectious viruses. At this concentration, the time courses of protein synthesis in HSV-1-infected HeLa cells with and without atropine were similar. Therefore, this experiment suggests that translation is not the target of the action of atropine. Moreover, since both the level and the pattern of the polypeptides synthesized late were the same whether atropine was present or not, this result suggests that neither the transcription of viral mRNAs nor the replication of viral DNA seems to be affected by this compound. Moreover, incorporation of [^{3}H]uridine and [^{3}H]thymidine into infected cells was at control levels under 400 μg of atropine per ml (data not shown).

Next, we analyzed the effect of atropine on the glycosylation of viral proteins. For this purpose, HSV-1-infected cells were labeled with [^{3}H]glucosamine from 4 to 16 h postinfection, and the proteins were separated by polyacrylamide gel electrophoresis. Figure 3A shows the autoradiogram of such a gel, where 200 μg of atropine per ml completely suppressed the glycosylation of viral proteins. Figure 3B shows the time course of [^{3}H]glucosamine incorporation into HSV-1-infected cells in the presence or absence of 200 μg of atropine per ml. Remarkably enough, the peak of viral glycosylation was abolished by atropine. Since at those concentrations, atropine had no effect on translation, this suggests that atropine acts by inhibiting the glycosylation of viral proteins.

Analysis of HSV-1 virions formed in atropine-treated cells. To test whether new virions were formed in atropine-treated cells, we infected HeLa cells with HSV-1 in the absence or presence of 200 μg of atropine per ml. The virions formed were collected and analyzed under the electron microscope. Table 1 shows that in the presence of 200 μg of atropine per ml there was lower reduction in the amount of virion particles formed than in the amount of infectious particles, and the percentages of the four morphological types obtained are similar both in control and in atropine-treated cells.

The infectivity of these virions was tested by adding the same volume of the HSV-1 preparation and analyzing the level of protein synthesis 18 h after its addition to new cultures of HeLa cells. Figure 4A shows that there was a ca. 1/50 to 1/100 reduction in the infectivity of the virions obtained from atropine-treated cells. These numbers are in good agreement with the reduction previously observed in Fig. 1. When we infected cells with the same volume of virus grown in control HeLa cells or in cells treated with atropine and analyzed the time course of protein synthesis, almost no viral proteins were apparent in cells infected with virus obtained from atropine-treated cells (Fig. 4B). Some large proteins in the high-molecular-weight region started to appear at 10 to 12 h postinfection with virions obtained from atropine-treated cells, indicating that they are less infectious as compared with control virions.

Taken together, these results suggest that virions formed

TABLE 1. Effect of atropine on the formation of HSV-1 virions

Treatment	PFU/ml	% Total particles	Particle type (%)			
			Naked		Enveloped	
			Empty	Full	Empty	Full
Mock infected	3×10^7	100	35	51	5	8
Atropine (200 μg/ml)	9×10^5	48	46	43	5	6

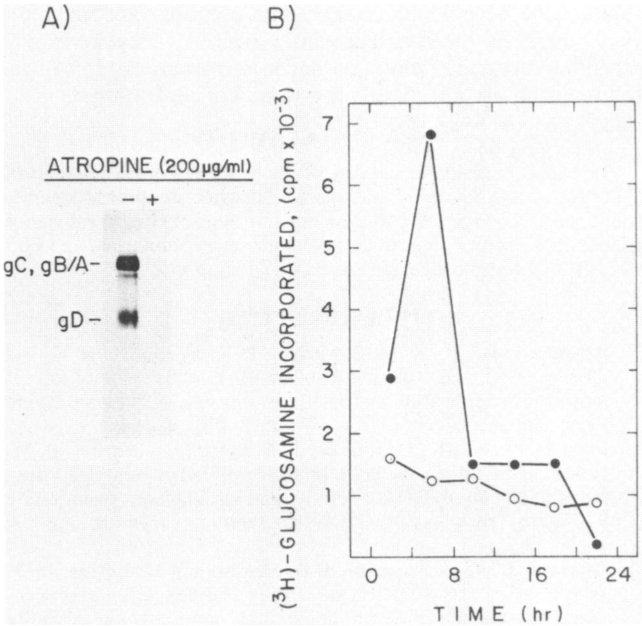

FIG. 3. Effect of atropine on protein glycosilation in HSV-1-infected cells. (A) Pattern of glycosilation in HSV-1-infected cells that were untreated or treated with 200 µg of atropine per ml from the beginning of infection. Glycoproteins were labeled with [^{3}H]glucosamine from 4 to 16 h postinfection. gC, gB/A, and gD are HSV-1 glycoproteins. (B) Time course of [^{3}H]glucosamine incorporation in HSV-1-infected cells that were either untreated (closed circles) or treated with 200 µg of atropine per ml (open circles) from the beginning of infection. Pulses were given at 4-h intervals.

in atropine-treated cells are defective and do not infect cells in a second round of infection. According to the results reported in Fig. 3, it is possible that these virions could have nonglycosylated proteins. To test this, virions grown in control or atropine-treated cells were labeled with [^{35}S]methionine or with glucosamine, and their protein composition was analyzed by polyacrylamide gel electrophoresis. Figure 5 shows that the virions thus obtained contained a similar level of [^{35}S]methionine label, irrespective of their source, but those grown in the presence of atropine did not have their proteins glycosylated. This result further strengthens the idea that atropine acts by inhibiting protein glycosylation.

Structure-activity relationship of atropine. Atropine [endo (±)-α-(hydroxymethyl)benzene-acetic acid, 8-methyl-8-azabicyclo[3.2.1]oct-3-yl ester, or 1α-H-5α-H-tropan-3α-ol(±)-tropate] comes from the esterification of one molecule of tropic acid with one molecule of tropine. Neither tropine nor tropic acid was as active as atropine in the antiherpesvirus effect. They did not reduce the cytopathic effect (CPE) at 400 µg/ml (data not shown). Neither were the related compounds, scopolamine [6β,7β-epoxy-1αH, 5αH-tropan-3α-ol-(−)tropate], eucatropine (α-hydroxybenzeneacetic acid, 1,2,2,6-tetramethyl-4-piperidinyl ester), and homatropine (1αH, 5αH-tropan-3α-ol-mandelate), active at 400 µg/ml. It should be pointed out that atropine methylbromide is devoid of antiherpesvirus action, perhaps because atropine methylbromide is a charged molecule and cannot cross cellular membranes.

DISCUSSION

The finding that several natural compounds have antiherpesvirus activity (Alacarón et al., in press) has prompted us to investigate their mechanism of action in an attempt to provide new inhibitors of viral functions. Atropine is an inhibitor that shows significant antiherpesvirus action at 200 µg/ml. Although this concentration seems rather high, particularly when compared with the activity of some nucleoside analogs, such as acyclovir or bromovinyl deoxyuridine, it is lower than the amount necessary for phosphonoformate or 2-deoxyglucose to exert a comparable inhibitory effect on HSV-1 (Alarcón et al., in press). It still remains to be tested whether atropine acts in the same way against HSV-1 infections in animals. Nevertheless, the main goal of our study was to clarify the step blocked by atropine during virus replication. According to our results, the action of atropine is clearly located at the level of protein glycosylation. The number of inhibitors discovered that block protein glycosylation has greatly increased during the last few years (4, 7, 8). Some of them, like tunicamycin or 2-deoxy-D-glucose, block glycosylation at a very early step, when the activated sugar is donated to dolichol-phosphate, whereas others, like nojirimycin, bromoconduritol, or swainsonine, affect the trimming of the oligosaccharide attached to the protein. We do not yet know exactly which step in glycosylation is blocked by atropine, but it is most probably before the oligosaccharide is donated to the protein, because the viral proteins are not labeled in the presence of [^{3}H]glucosamine.

Of particular interest is the finding that virions are assembled even though the viral proteins are not properly glycosylated, suggesting that glycosylation is not a prerequisite for this to take place. In fact, it has been reported recently that underglycosylated HSV-1 glycoproteins synthesized in the

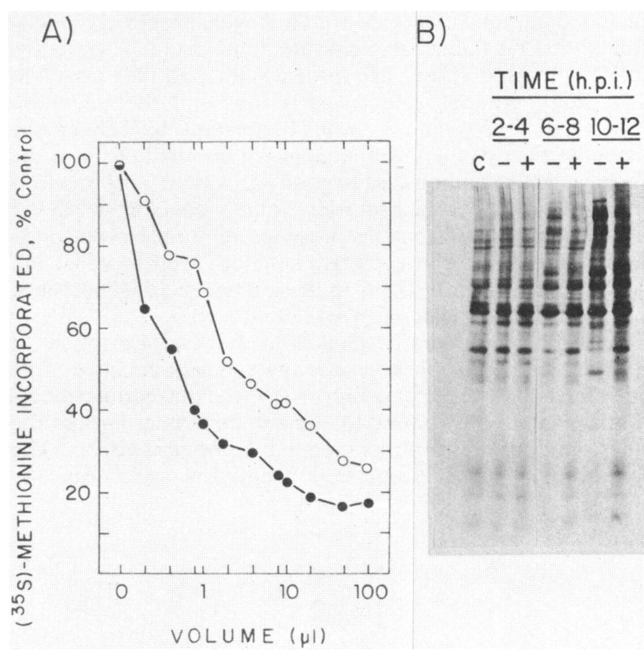

FIG. 4. Production of defective HSV-1 virions in the presence of atropine. (A) HeLa cells were infected with HSV-1, and at 24 h postinfection HSV-1 particles were obtained that were either untreated (closed circles) or treated with 200 µg of atropine per ml (open circles). Different volumes of these extracts were used to infect HeLa cell monolayers. Protein synthesis was estimated at 18 h postinfection. (B) Time course of protein synthesis in HeLa cells infected with extracts obtained from HSV-1-infected HeLa cells that were either untreated or treated with atropine at 200 µg/ml. The numbers indicate the times postinfection when two hourly pulses with [^{35}S]methionine were given. Lane C, Uninfected control cells.

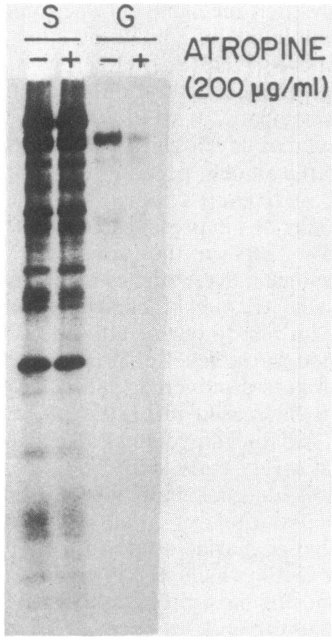

FIG. 5. Effect of atropine on the synthesis of virion polypeptides and glycoproteins. HSV-1-infected HeLa cells were labeled with [^{35}S]methionine (lane S) or [^{3}H]glucosamine (lane G) in the presence or absence of atropine. Virions were obtained and electrophoresis was carried out as described in the text.

presence of 2-deoxy-D-glucose are normally transported to the cellular membrane and they can elicit complement-mediated immune cytolysis (3). However, proper glycosylation is vital for the subsequent infectious cycle. Thus, when nonglycosylated virions are formed, although they attach to cells, they are not able to carry on with the infection. Therefore, glycosylation seems important to deliver the virion particle or the virion genome in the cytoplasm or the nucleus. These findings are in good agreement with previous results from other laboratories. Thus, enveloped HSV-1 particles are formed in the presence of tunicamycin or 2-deoxy-D-glucose. These particles adsorb well to cells, but the transport of virus DNA to the nucleus is blocked, since they are so much less infectious (9, 10).

Regarding the potential clinical usefulness of atropine, it does not seem feasible to use it systemically because of its side effects. However, it is known that the muscarinic effects of atropine are only bound to one optical isomer but not the other, and perhaps both isomers have antiviral effects. The possibility of using atropine as a antiviral agent topically should also be pursued, since some compounds that differ very much in their potency in vitro, like acyclovir and phosphonoformate, show antiherpesvirus activity in topical treatment in vivo at almost the same concentration.

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